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Interstitial chromatin alteration causes persistent p53 activation involved in the radiation-induced senescence-like growth arrest

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Abstract

Various stresses including ionizing radiation give normal human fibroblasts a phenotype of senescence-like growth arrest (SLGA), manifested by p53-dependent irreversible G1 arrest. To determine the mechanism of persistent activation of p53, we examined phosphorylated Ataxia telangiectasia mutated (ATM) and phosphorylated histone H2AX foci formation after X-irradiation. Although the multiple tiny foci, detected soon after (<30 min) irradiation, gradually disappeared, some of these foci changed to large foci and persisted for 5 days. Large foci containing phosphorylated ATM and γ-H2AX co-localized and foci with p53 phosphorylated at serine 15 also showed the same distribution. Interestingly, the signals obtained by telomere fluorescence in situ hybridization (FISH) assay did not co-localize with 90% of the large foci. Our results indicate that chromatin alteration in interstitial chromosomal regions is the most likely cause of continuous activation of p53, which results in the induction of SLGA by ionizing radiation.

Keywords: Senescence-like growth arrest; Premature cellular senescence; Persistent p53 activation; Histone H2AX; ATM; Telomere; Focus formation; Ionizing radiation

Previously, Hayflick and Moorhead [1,2] reported that cellular senescence depended on cellular division potentials of somatic cells in vitro. Senescent human diploid cells differ from proliferating cells in that they show p53-dependent irreversible growth arrest, large and flat cell morphology, and the expression of senescence-associated β-galactosidase (SA-β-gal), etc. On the other hand, it has been found that various external stresses, including exposure to X-rays, cause the senescent phenotype to arise prematurely in normal human diploid cells. Normal human diploid cells irradiated with X-rays show p53-dependent irreversible growth arrest, known as senescence-like growth arrest (SLGA) [3]. It is well known that ionizing radiation causes p53 expression levels to increase and initiates phosphorylation of p53 at serine (Ser) 15, accumulation and phosphorylation of p53 being important to activate p53 as a transcriptional factor [4,5]. In fact, cells with induced SLGA have been found to show a level of p53 accumulation and phosphorylation similar to that found in senescent cells, as well as the subsequent induction of p21 expression, a known downstream gene regulated by p53 [3]. It has been shown that SLGA is induced significantly in primary human fibroblasts 5 days after irradiation with X-ray doses greater than 4 Gy. During these responses, p53 continues accumulating via ser15 phosphorylation during SLGA-expression, contributing to persistent and irreversible checkpoint activation.

It is believed that irreparable DNA damage caused by SLGA manifests itself in the form of DNA double-strand breaks, since this type of damage is lethal and

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activates p53 [6]. Additionally, it is thought that telomere status associates with the induction of cellular senescence [7,8]. Telomere structure consists of telomeric DNA and telomere repeats binding factor (TRF) proteins, and the telomeric DNA forms a telomere-loop (t-loop) with TRF masking the DNA double-strand ends [9,10]. TRF2, which is one of the TRF proteins, plays an essential role in the formation of t-loops and a t-loop never forms without TRF2 in vitro [10]. Although it is not yet known how telomeres are involved in cellular senescence, it has been thought that shortened telomere, which resembles DNA double-strand breaks, is associated with the induction of cellular senescence. Telomeric DNA is shortened with every cell division, and the average telomere length of 12 kb in normal human diploid cells is reduced to an average of 5 kb in senescent cells [11,12]. Because the expression of hTERT, which is a catalytic subunit of telomerase, extends the lifespan by inhibiting telomere shortening [7], it is highly possible that shortened telomeres appear to be unable to form a t-loop. As a result, exposed DNA ends, which are similar to DNA double-strand breaks by ionizing radiation, then cause persistent p53 activation. It is expected that unreparable DNA double-strand breaks cause SLGA, however, telomere shortening has not been observed [3]. Nevertheless, the induction of premature cellular senescence was recently demonstrated in a study with a TRF2 mutant ($TRF2^{\Delta B \Delta M}$), which is a deleted C-terminus of TRF2 and cannot form a t-loop since it loses the ability to bind to telomeric DNA [8,13]. These data suggest that cellular senescence is induced by dysfunctional telomeres due to an unwinding t-loop, but not merely due to telomeric shortening. This finding also suggests the possibility that dysfunctional telomeres caused by ionizing radiation, but not telomere shortening, are associated with SLGA.

Recent developments in molecular biology allow us to detect DNA double-strand breaks sensitively and efficiently, using the detection of histone H2AX phosphorylation [14–16]. Histone H2AX at Ser139 is phosphorylated by ATM (forming γ-H2AX) in response to ionizing radiation or by other inducers of DNA double-strand break. ATM also autophosphorylates itself at Ser1981, a process dependent on DNA double-strand break [17,18]. γ-H2AX forms foci within a few minutes after X-irradiation, and these foci co-localize with various DNA repair proteins, such as the MRE11/Rad50/NBS1 (MRN) complex, Rad51 and others [19,20]. Furthermore, comparison of the number of γ-H2AX foci with the number of DNA double-strand breaks shows that γ-H2AX foci are always associated with DNA double-strand breaks [22].

In the present study, we used γ-H2AX foci as a marker of DNA double-strand breaks and examined its inter-nuclear distribution of DNA damage associated with persistent p53-activation in SLGA. Also we examined whether or not the localization of γ-H2AX foci was related to the telomeres within damaged cells.

Materials and methods

Cell cultures and X-irradiation. Normal human diploid cells, HE49, were exponentially grown in Eagle's minimum essential medium containing 10% fetal bovine serum in a 5% CO_2 incubator at 37 °C [23]. Cells were irradiated with 4 Gy of X-rays from an X-ray generator at 150 kVp and 5 mA with a 0.1-mm copper filter. X-irradiation was administered at a rate of 0.492 Gy/min.

Immunofluorescence assay. Cells grown on coverslips to 60–70% confluence were washed once with cold cytoskeleton (CSK) buffer, fixed with 4% paraformaldehyde in CSK buffer for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 in CSK buffer for 5 min on ice. As another fixative method which removes nucleoplasmic proteins or the proteins associated to chromatin loosely, following permeabilization with 0.5% Triton X-100 in CSK buffer for 2 min on ice, cells were fixed with 4% paraformaldehyde in CSK buffer for 20 min at room temperature and then treated with 0.5% NP-40 in CSK buffer for 5 min at room temperature. The cells were then incubated for 2 h at 37 °C with mouse monoclonal anti-phosphorylated H2AX at Ser139 antibody (Upstate Biotechnology, Lake Placid, NY, USA), rabbit polyclonal anti-phosphorylated H2AX at Ser139 antibody (Upstate Biotechnology), mouse monoclonal anti-p53 (Lab Vision, Fremont, CA, USA), rabbit polyclonal anti-phosphorylated p53 at Ser15 (Cell Signaling Technology, Beverly, MA, USA) or rabbit polyclonal anti-phosphorylated ATM at Ser1981 antibody (Rockland, Philadelphia, PA, USA) at a dilution of 1:10000, 1:200, 1:2000, 3:100 or 1:10000 in Tris-buffered saline (TBS), respectively. After being washed with phosphate-buffered saline (PBS^-) three times, the cells were incubated for 1 h at 37 °C with Alexa Fluor 488-conjugated goat anti-mouse or rabbit antibody or Alexa Fluor 594-conjugated anti-mouse or rabbit antibody (Molecular Probes, Eugene, OR, USA) at a dilution of 1:1000 in TBS. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 10 ng/ml in TBS; Molecular Probes, Eugene, OR, USA) for 30 min, and coverslips were mounted on slide glasses with PBS^- containing 10% glycerol. Images were acquired with an Olympus fluorescence microscope and then analyzed with IP Lab software (Scanalytics, Fairfax, VA, USA).

Immuno FISH. Immuno FISH assay was performed as described by Herbig et al. [24]. Briefly, cells were fixed, permeabilized, and stained with anti-phosphorylated histone H2AX antibody as described above. After staining, labeled protein was cross-linked with 4% paraformaldehyde in PBS^- for 20 min at room temperature. The samples were then dehydrated in 70%, 90%, and 100% ethanol for 3 min each and air-dried, and DNA was denatured for 30 min on a hotplate at 80 °C. After hybridization with a telomere-PNA probe for 5 h, the cells were washed three times with 70% formamide/10 mM Tris (pH 6.8) for 15 min, followed by a 5-min wash with 0.05 M Tris/0.15 M NaCl (pH 7.5)/0.05% Tween 20 and a 5-min wash with PBS^-. Mounting and microscopic analysis were performed as in immunofluorescence assay.

SA-β-gal staining. SA-β-gal staining was carried out as described by Dimri et al. [25]. Briefly, cells were washed once with PBS^- and fixed with 2% paraformaldehyde containing 0.2% glutaraldehyde for 5 min at room temperature. After fixation, cells were washed extensively with PBS^- and were then incubated with stain solution (40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal).

Results

Phosphorylated ATM and γ-H2AX foci formation during induction of SLGA

Normal human diploid cells irradiated with 4 Gy of X-rays, a dose that results in ~3% survival, initiating showing a similar expression of SA-β-gal to that of

unirradiated cells. However, expression was induced beginning at 3 days after irradiation, and approximately 60% of irradiated cells were found to be positive for SA-β-gal 5 days after irradiation. During this time, the cell morphology changed, leaving cells large and flat non-dividing cells (data not shown). Although both γ-H2AX and phospho-ATM were rarely detected in unirradiated cells, these formed foci. And most of both foci do not coexist (<10%) (Fig. 1). Co-localized phosphorylated ATM and γ-H2AX foci appeared as tiny foci within 30 min after irradiation, and most of these foci disappeared rapidly within 20 h after irradiation. It was noted that the size of the foci became larger beginning at 4 h after irradiation and peaking at 20 h after irradiation (Fig. 1). Most of the residual foci detected at 5 days after irradiation were large (Fig. 1). There was no difference in size or the number of residual foci between 2 and 5 days after irradiation. At 5 days post ionizing radiation had an average of 3 co-localized large foci per cell (Fig. 1).

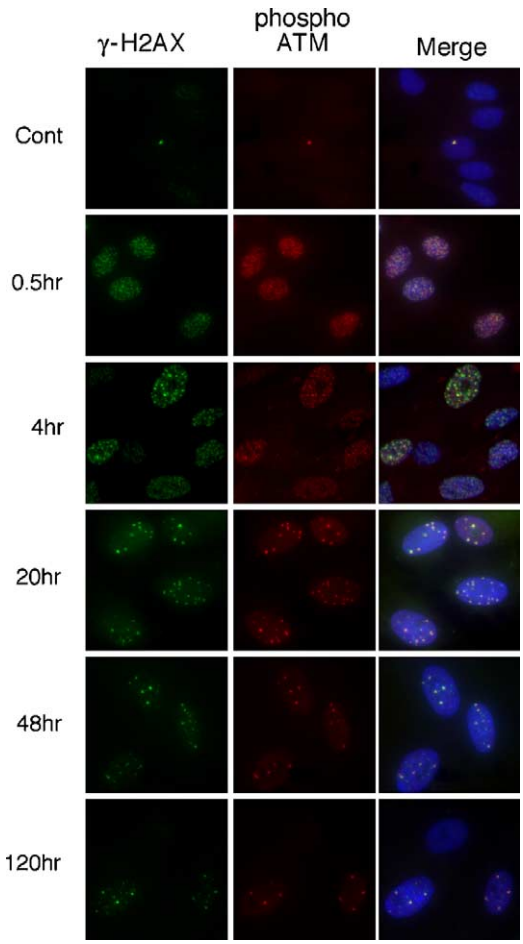

Fig. 1. γ-H2AX and phosphorylated ATM foci in cells irradiated with 4 Gy of X-rays. Cells were dual-stained with anti-γ-H2AX at Ser139 (green) and anti-phosphorylated ATM at Ser1981 (red). Cell nuclei were counterstained with DAPI (blue).

Localization of p53 after X-irradiation

Our examination of p53 immunofluorescence revealed that p53 was detected in 30% of unirradiated cells, and that these cells were observed independently of the presence of γ-H2AX foci (Fig. 2 and Table 1). In cells with γ-H2AX foci, p53 foci were co-localized. After X-irradiation, the nuclei of approximately half of the irradiated cells were p53-positive at 4 h, and the proportion of p53-positive cells increased thereafter. At 5 days after irradiation, more than 90% of cells were positive for the p53 signal (Table 1). p53 is present as not dot but focus in the cell. The frequency of cells with p53 foci did not change until 4 h after X-irradiation, wherein it increased and reached approximately 50% at 20 h after irradiation. Half of p53-positive cells had formed p53 foci by 5 days after irradiation (Table 1). All p53 foci observed at 5 days after irradiation had co-localized only with large foci of γ-H2AX.

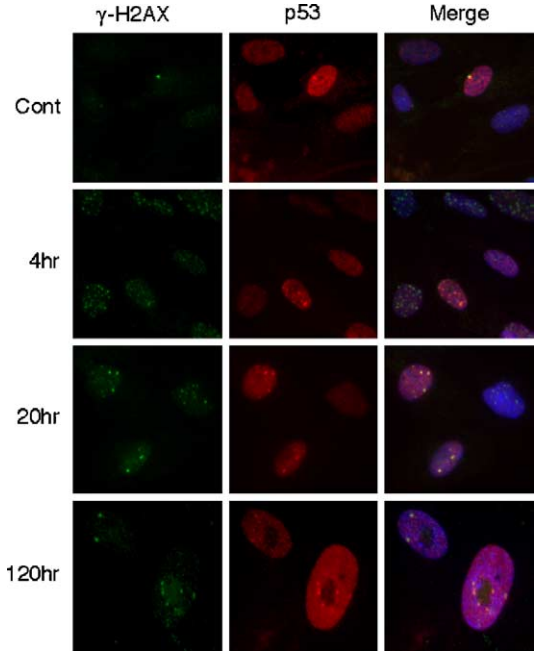

Fig. 2. γ-H2AX and p53 cells irradiated with 4 Gy of X-rays. Cells were dual-stained with anti-γ-H2AX at Ser139 (green) and anti-p53 (red). Cell nuclei were counterstained with DAPI (blue).

Table 1
Proportion of positive cells for γ-H2AX foci, p53 accumulation and p53 foci during SLGA-induction

Cells	% Positive cells		
	γ-H2AX[a]	p53	p53 foci
Control	5.1 (5.1)	30.0	1.7
4 h after IR	99.5 (13.2)	48.5	1.5
20 h after IR	80.0 (71.5)	66.1	23.2
120 h after IR	70.1 (70.1)	97.5	49.2

[a] The number in parenthesis showed proportion of large γ-H2AX foci in counted cells.

p53 phosphorylation during the induction of SLGA

We examined the phosphorylation of p53 at Ser15 in the same time-course as that described in the p53 localization study above. Phosphorylated p53 was detected in 10% of unirradiated cells and showed two types of localization: almost all unirradiated positive cells showed dispersed localization, and the remaining unirradiated positive cells showed a focus (Fig. 3A and "FT" in Table 2). Phospho-p53 foci appeared saliently at 4 h after irradiation, but dissociated and diffused in more than half of foci-positive cells thereafter. There were no phospho-p53 foci co-localized with γ-H2AX foci after 5 days post-irradiation. Using nuclear extraction methods, in which cells were treated with detergent before fixation to remove nucleoplasmic proteins or proteins associated with chromatin loosely, revealed a gradual increase in cells positive for phosphorylated p53 foci after irradiation, and almost all phosphorylated p53 foci detected by this method localized with γ-H2AX foci (Fig. 3B and "TFN" in Table 2).

Telomere-independent localization of γ-H2AX foci in cells with induced SLGA

We examined the localization of γ-H2AX foci to telomere in interphase cells with induced SLGA. Immuno FISH assay showed that γ-H2AX foci did not localize at telomere regions at all in unirradiated cells. Additionally, only 10% of γ-H2AX foci were observed near (arrowhead in the middle panel of Fig. 4) or on the telomere signal (arrowhead in the bottom panel of Fig. 4) in cells with induced SLGA. In general, most foci did not show co-localization with the telomere signal.

Discussion

In the present study, we found that γ-H2AX foci, observed at a rate of 3 foci per cell, remained in 70% of irradiated cells, and all of them co-localized with phosphorylated ATM foci even at 5 days after irradiation (Fig. 1 and Table 1). It has been reported that ATM is activated by autophosphorylation at Ser1981 and activated ATM phosphorylates histone H2AX at the site of DNA double-strand breaks in response to ionizing radiation [17,18]. In another study, it was found that γ-H2AX foci co-localized with various repair proteins of DNA double-strand breaks [19,20], assuming that DNA double-strand breaks remain in cells with induced SLGA. Other studies have demonstrated that both γ-H2AX formation and ATM activation can occur without DNA damage, but the foci look different and more diffuse [21]. Interestingly, we also found in the present study that only large phosphorylated-ATM/γ-H2AX co-localized foci, whose size differed from that of the tiny foci observed 30 min after irradiation, remained at 5 days after irradiation (Fig. 1). These large foci present thereafter were observed beginning at 4 h after irradiation in 13% of irradiated cells, and almost all foci became large within 20 h after irradiation. Although the biological significance of large γ-H2AX foci remains unknown, one possible expla-

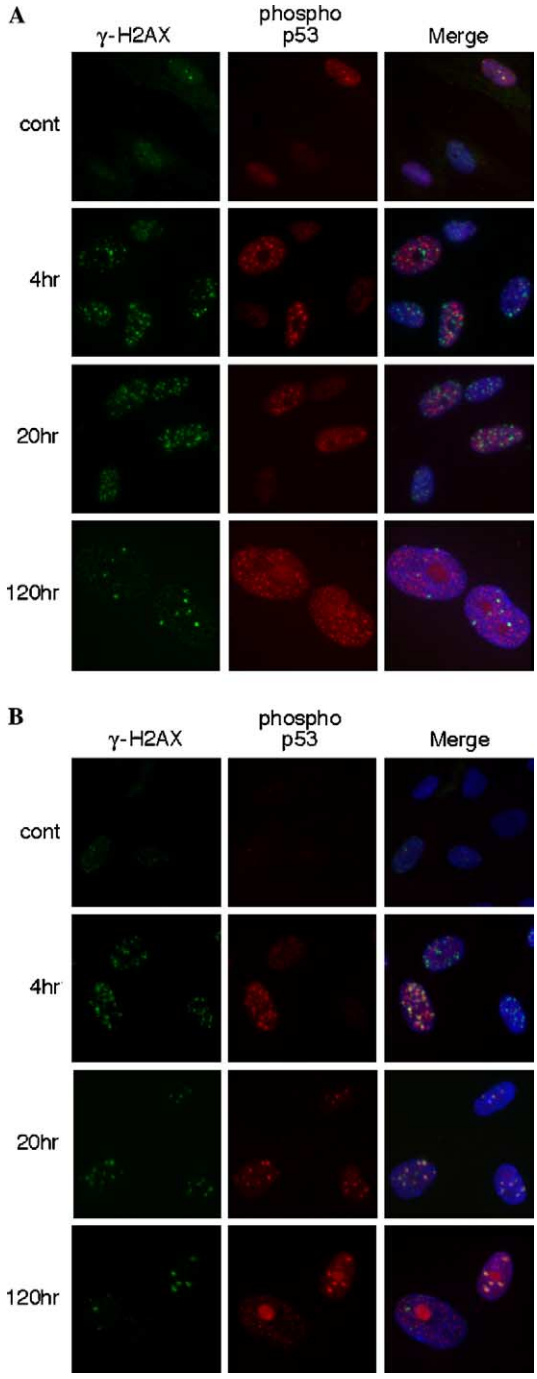

Fig. 3. γ-H2AX and phosphorylated p53 (phospho-p53) in cells irradiated with 4 Gy of X-rays. Cells were dual-stained with anti-γ-H2AX at Ser139 (green) and anti-phosphorylated p53 at Ser15 (red). Cell nuclei were counterstained with DAPI (blue). (A) Cells were fixed with 4% paraformaldehyde in CSK buffer and permeabilized with 0.5% Triton X-100 in CSK buffer. (B) Cells were subjected to nuclear extraction to remove nucleoplasmic proteins or the proteins associated to chromatin loosely. In phosphorylated p53 images at 120 h after irradiation, the larger red spot located near the center of each cell represents a nucleolus but not a phosphorylated p53 focus.

Table 2
Frequency of phosphorylated p53 (phospho-p53) positive cells during SLGA induction

Cells	% Positive cells					
	FT[a]			TFN[b]		
	Phospho-p53	Dispersion	Focus[c]	Phospho-p53	Dispersion	Focus[c]
Control	10.4	9.8	0.6 (0.6)	1.3	0	1.3 (1.3)
4 h after IR	95.2	18.4	76.8 (5.9)	94.1	78.9	15.2 (11.2)
20 h after IR	96.1	69.6	26.5 (2.0)	97.2	67.0	30.2 (25.2)
120 h after IR	100	79.7	20.3 (2.4)	89.6	61.8	27.3 (26.9)

[a] FT indicated fixative method with 4% paraformaldehyde-0.5% Triton X-100 (detail shown in Material and methods).
[b] TFN indicated fixative method with 0.5% Triton X-100-4% paraformaldehyde-0.5% NP-40 (detail shown in Material and methods).
[c] The number in parenthesis showed the frequency of phosphorylated p53 foci colocalized with γ-H2AX foci in counted cells.

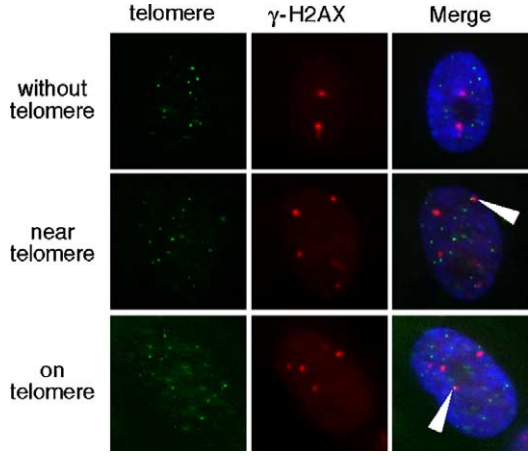

Fig. 4. Localization of large γ-H2AX foci (red) and telomeres (green) in cells with induced SLGA. The arrowhead in the middle panel indicates large γ-H2AX foci located near the telomere signal, and the arrowhead in the bottom panel indicates those located on the telomere signal.

nation is that the large foci represent non-repaired or potentially irreparable DNA double-strand breaks. However, our recent study demonstrated γ-H2AX foci on rejoined chromosomes (in press). Therefore, it is also possible that large γ-H2AX foci reflect chromatin alteration after DNA rejoining. It has been reported that the chromatin remodeling factors included in ionizing radiation-induced foci (IRIF) consistent with γ-H2AX [26], various repair proteins of DNA double-strand break-, and checkpoint-associated proteins. For example, ATM is autophosphorylated not only in DNA double-strand breaks but also in chromatin alteration [18]. Thus, our data provide evidence in favor of the idea that X-irradiation with 4 Gy causes 70% of irradiated cells to suffer three instances of irreparable chromatin damage per cell after 5 days.

We examined p53 activation in cells positive for large γ-H2AX foci, finding that the levels of phosphorylated p53 and expression level of this protein is detected by the immunofluorescence method were much higher than the control level in large foci-positive cells (Figs. 2 and 3A). Persistent p53 activation determined by immunoblotting has been reported in our previous study [3]; in addition, we show clearly here that p53 is persistently activated in cell, which has irreparable damage. We also show that co-localization of p53 foci and large γ-H2AX foci begins at 20 h after X-irradiation. Furthermore, phosphorylated p53 at Ser15 foci appeared and co-localized only with large γ-H2AX foci after fixation with the nuclear extraction method (Fig. 3B). These results suggest that persistent chromatin damage as shown by the large size of γ-H2AX foci causes persistent ATM activation, resulting in persistent p53 activation observed in SLGA.

If we assume that it is true that dysfunctional telomere is a cause of cellular senescence, it is possible that ionizing radiation-induced telomere dysfunction is associated with SLGA induction; indeed, ionizing radiation is known to cause telomere dysfunction following telomere instability in normal and radiosensitivity-relative protein-deficient cells [27–29]. It is thought that telomere shortening causes telomere instability [30], but telomere shortening was not observed in cells with induced SLGA in our previous study [3]. In contrast, a recent study with TRF2$^{\Delta B\Delta M}$ revealed that dysfunctional telomere that does not form a t-loop, indicating about but not telomere length induces telomere instability and premature cellular senescence [8]. We, therefore, examined the relationship between the localization of large γ-H2AX foci and telomere positions. The present immuno FISH assay showed that approximately 90% of large foci of γ-H2AX did not co-localize with telomeres, suggesting that persistent p53 activation occurs in the interstitial chromosomal region.

In the present study, ionizing radiation-induced large-scale and irreparable damage in the interstitial chromosomal region was found to be the most likely cause of continuous activation of p53, although p53 is activated transiently by the formation of DNA double-strand breaks. Our previous report suggests the possibility that ionizing radiation-induced initial damage in the interstitial chromatin regions may affect telomere stability in several generations after irradiation [27]. It is suggested that irreparable damage, which manifests itself as large γ-H2AX foci may be associated with the later expression of telomere instability, and SLGA induction plays a role in the suppression of telomere dysfunction following genomic instability in delayed response to ionizing radiation.

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